New Sources of Dietary Myosmine Uptake from Cereals, Fruits, Vegetables, and Milk

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Myosmine has been regarded as a specific tobacco alkaloid until investigations pointed out that nuts and nut products constitute a significant source of myosmine. In the present study it is shown that the occurrence of myosmine is widespread throughout a large number of plant families. Using a method for extraction practicable for all examined foods, quantitative analysis through internal standard addition showed nanograms per gram amounts. Positively tested edibles were staple foods such as maize, rice, wheat flour, millet, potato, and milk and also cocoa, popcorn, tomato, carrot, pineapple, kiwi, and apples. No myosmine was detectable in other vegetables and fruits such as lettuce, spinach, cucumber, onion, banana, tangerines, and grapes. Myosmine is easily nitrosated giving rise to a DNA adduct identical to the esophageal tobacco carcinogen *N*-nitrosonornicotine. Therefore, the role of dietary myosmine in esophageal adenocarcinoma should be further investigated.

KEYWORDS: Dietary myosmine uptake; staple foods; cereals; vegetables; fruits; milk; esophageal adenocarcinoma

INTRODUCTION

The occurrence of myosmine in nuts and nut products was shown by Zwickenpflug et al. (*1*). Nitrosation of myosmine yields not only *N*-nitrosonornicotine, an esophageal carcinogen in rats, but to a greater extent 4-hydroxy-1-(3-pyridyl)-1-butanone, presumably through a one-step reaction leading to a reactive intermediate, an unstable diazotate (*2*). Under nitrosation conditions adduct formation of myosmine with DNA or proteins has been verified in vivo and in vitro by GC-MS after hydrolysis of the adduct and derivatization of the resulting 4-hydroxy-1-(3-pyridyl)-1-butanone (*3*). Optimal reaction conditions for in vitro nitrosation are at pH 2−5, yielding high amounts of 4-hydroxy-1-(3-pyridyl)-1-butanone (*4*). This aspect may explain some epidemiologic studies correlating esophageal adenocarcinoma with gastroesophageal reflux. The reflux of gastric juice is associated with a lower pH in the esophagus and increased possibility of nitrosation. The risk for adenocarcinoma (AC) can exceed 40-fold for long-term sufferers with severe symptoms (*5*). Gastroesophageal reflux disease (GERD) may be related to changing eating habits and psychosocial stress (*6*). Although the sequence of GERD leading to intestinal metaplasia of the gastroesophageal junction, the so-called Barrett esophagus (BE), and finally to AC is well established, the incidence of AC is still low in patients with BE and other factors such as diet play a decisive role (*7*).

The type of tumor most commonly found in the esophagus has changed in the past 40 years. In the past, 90% of all tumors were of the squamous cell type (SCC), but the incidence of esophageal AC has increased in recent decades. Today it is predominant to the squamous cell type in the United States and in Europe (*8*). In contrast to SCC, AC of the esophagus correlated with body mass index but not with tobacco and alcohol consumption (*9−13*). The rising incidence of AC parallels the increased prevalence of obesity in the highly industrialized Western countries. Some food and dietary components play an important role in esophageal cancer. Epidemiologic studies in eastern Nebraska point to a higher risk for diets high in meat, salty snacks, milk, and white bread (*10*). A case-control study in Venezuela showed an increased risk with rising consumption of cereals, starchy vegetables, dairy products, and fruit (*14*). A higher risk for cancer of the esophagus has been associated also with increasing intake of refined cereals (bread, pasta, and rice) (*15*). On the other hand, diets rich in fruits and vegetables could lower the risk of esophageal adenocarcinoma to ∼50% (*10*, *16*). In addition, uptake of vitamin A, vitamin C, and crude fiber was inversely associated with esophageal adenocarcinoma (*17*). However, the association between dietary factors and risk of esophageal adenocarcinoma is not yet well understood. The no longer "tobacco specific" alkaloid myosmine [3-(1-pyrrolin-2-yl)pyridine] could be a unifying element to connect the different findings.

The relevance of myosmine would be even greater, if its occurrence was not limited to only tobacco and nuts. Therefore, more dietary components have been analyzed for myosmine. The sample preparation has been modified in comparison to the nut samples, and quantification was performed by adding an internal standard, D$_4$-myosmine. Great importance has been attached to the choice of edibles. The most common staple foods

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Table 1. Myosmine Concentrations in Different Food and Dietary Components

food	myosmine[a] (ng/g) ± SD (n = 6)	range (ng/g)
rice, husked, raw	0.6 ± 0.4	0.19–1.15
maize, raw[b]	1.3 ± 0.4	0.48–1.60
popcorn, raw	4.8 ± 0.4	4.44–5.66
wheat flour, type 405, raw	0.4 ± 0.05	0.37–0.51
millet, raw	0.4 ± 0.01	0.41–0.43
potato, cooked, peeled	3.4 ± 0.7	2.41–4.47
tomato, raw	0.6 ± 0.05	0.53–0.66
cocoa, raw[c]	0.8 ± 0.06	0.68–0.85
milk (3.5% fat)	1.2 ± 0.07 (ng/mL)	1.16–1.35
cream (30% fat)	6.1 ± 1.2	5.93–6.26
carrot, raw	0.02 ± 0.01	0.01–0.03
apple, raw, peeled	1.2 ± 0.04	1.12–1.25
pineapple, raw, peeled	0.7 ± 0.03	0.66–0.72
kiwi, raw, peeled	1.4 ± 0.2	1.15–1.61

[a] Based on wet weight. [b] Commercial powder for polenta preparation. [c] Commercial powder for bakery and instant milk drinks.

as well as fruits, vegetables, and other interesting foods such as cocoa and milk have been analyzed.

The intention of this work was not to give a detailed overview about different brands on the market but to show the widespread occurrence of myosmine in plants. In combination with other risk factors for esophageal adenocarcinoma, myosmine uptake via food may be another important detail in this complex carcinogenic process.

MATERIALS AND METHODS

Chemicals for synthesis of myosmine were obtained from Sigma-Aldrich (Deisenhofen, Germany). D_4-Myosmine was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). All solvents and chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany). Samples of fresh fruits and vegetables were purchased from local food stores. All other samples were from commercial brands purchased at retail during 2001/2002 (**Table 1**).

Synthesis of Myosmine. Myosmine was synthesized according to the method of Brandänge and Lindblom (*18*). Briefly, ethyl nicotinate (0.18 mol) and *N*-vinylpyrrolidone (0.18 mol) were added dropwise to a solution of sodium hydride (0.26 mol, 10.4 g of a 60% mineral oil suspension) in dry toluene (100 mL). After 2.5 h of refluxing, the solution was treated with 280 mL of a mixture of H_2O/concentrated HCl (50:90, v/v) and adjusted with 10 N NaOH to pH 4. After separation from the toluene phase, the water phase was extracted three times with $CHCl_3$/EtOH (3:2, v/v) and dried over anhydrous Na_2SO_4. After concentration, the residue was treated with 250 mL of concentrated HCl and refluxed for 16 h. The reaction product was distilled under vacuum and yielded a caramel myosmine. The physicochemical parameters of the product were in accordance with values reported in the literature (*1*).

Sample Preparation. Samples of food, 50−60 g, were ground with a laboratory mill (Braun, Kronberg, Germany) or an Ultra-Turrax (Bachofer, Reutlingen, Germany). All samples except potatoes were analyzed in the raw state. Potatoes were peeled, after cooking, before they were cut in small pieces; apples, kiwis, pineapples, cucumbers, bananas, tangerines, and lemons had been peeled, and the peel was analyzed separately. Ten milliliters of 6 N H_2SO_4, 100 mL of H_2O, and 40 μL (308 ng) of a stock solution of D_4-myosmine in CH_2Cl_2 were added and the samples stirred for 16 h. After sonication for 3 min, the slurry was centrifuged at 1800g for 10 min. The supernatant was transferred into an Erlenmeyer flask. The sediment was suspended with 10 mL of H_2O, filtered (no. 520 B II^1/$_2$, Schleicher and Schuell, Germany), and combined with the supernatant. The extract was adjusted to pH 7 with 6 N NaOH and centrifuged at 1800g for 10 min. First, the supernatant was filtered (No. 602 H^1/$_2$, Schleicher and Schuell), then the sediment was resuspended with 5 mL of H_2O, and the

suspension was passed through the same filter. The filtrate was filtered a second time followed by adjustment of the pH to 7. The liquid was transferred to a preconditioned 3 mL C_{18} solid-phase extraction (SPE) tube with stainless steel frits and 500 mg of sorbent material 3CC/500 (Varian GmbH, Darmstadt, Germany). The extraction tube was washed with 3 volumes of H_2O. After being dried by centrifugation at 2200g, the tube was eluted with 4 × 500 μL of $CHCl_3$. The solution was concentrated at room temperature, and the residue was suspended with 70 μL of CH_2Cl_2 for analysis by GC-MS. In the analysis of carrot samples a thin layer chromatography (TLC) separation was executed on preparative Kieselgel F254 plates (Merck) using $CHCl_3$/MeOH (9:1, v/v). The relevant spots were removed from the plates, eluted with $CHCl_3$, and analyzed further by GC-MS.

Instrumentation. Analytical assessment of myosmine was performed in the electron impact (EI) mode at 70 eV on two different instruments. First, qualitative analyses were executed on an HP 5890 series II gas chromatograph coupled to an HP 5972 quadrupole mass selective detector (Hewlett-Packard, Waldbronn, Germany). The samples were separated on a 60 m × 0.2 mm i.d., 0.33 μm film thickness, DB5 capillary column (Phenomenex, Aschaffenburg, Germany). The temperature program was started at 80 °C, held for 1 min, and then raised at 15 °C/min to 325 °C, at which it was held for 5 min. The temperatures of the injection port, transfer line, and ion source were held at 280, 300, and 180 °C, respectively. Helium was used as the carrier gas in the constant flow mode with a head pressure of 20 kPa.

Later, a system consisting of a Fisons 8060 gas chromatograph coupled to a TRIO 1000 quadrupole mass selective detector (Fisons Instruments, Mainz, Germany) was used for quantification. Separation was executed on a 30 m × 0.25 mm i.d., 0.25 μm film thickness, ZB50 capillary column (Phenomenex), with helium as carrier gas (flow rate = 0.8 mL/min). A split/splitless injector was used in the splitless mode with an injection volume of 1 μL. The temperature program was started at 80 °C, held for 1 min, and then raised at 15 °C/min to 310 °C, at which it was held for 5 min. The temperatures of the injection port, transfer line, and ion source were held at 200, 300, and 250 °C, respectively.

All ions were monitored with dwell times of 100 ms. The acquisition in the scan mode for qualitative analyses was executed between m/z 50 and 200. In the SIM mode the masses m/z 51, 78, 105, 118, 122, 146, and 150 (122 and 150 for the internal standard D_4-myosmine) were recorded for quantitative analyses. All chromatograms and mass spectra shown in **Figures 1−3** have been generated with the second equipment.

Quantification of the Analytes and Recovery Rates. Quantification of myosmine was performed using D_4-myosmine as an internal standard. The limit of detection was found to be 15 ± 5 pg for both D_4-myosmine and for unlabeled myosmine. The response of the mass spectrometer was linear in the range of 10−1000 pg with a response factor for myosmine versus D_4-myosmine of ∼1. Within the limit of detection no background was apparent in routinely analyzed tap water samples. Recovery rates were between 51.6 and 90.5% depending on the food matrix.

RESULTS AND DISCUSSION

Figures 1 and **2** show GC-MS chromatograms and scans from standards of myosmine and D_4-myosmine, respectively. The data from a cream sample are displayed in **Figure 3**. Myosmine and D_4-myosmine were easily detected in the SIM mode with a retention time of 8.9 ± 0.1 min. In the scan mode the relative abundance of the characteristic mass fragments m/z 146/118 and 150/122 were identical in the standard and the sample extract.

Table 1 shows the amounts of myosmine found in various edibles. Myosmine levels ranged from 0.02 ng/g in carrots to 6.1 ng/g in cream. Other dietary components with a high content are popcorn and potatoes. The analysis of peels from selected fruits and vegetables showed no relevant divergence in the amounts of myosmine to the usually eaten rest of the fruits and vegetables (data not shown). An overview of all examined foods is given in **Table 2**, which includes also the corresponding plant families. The assessed samples were selected from consideration
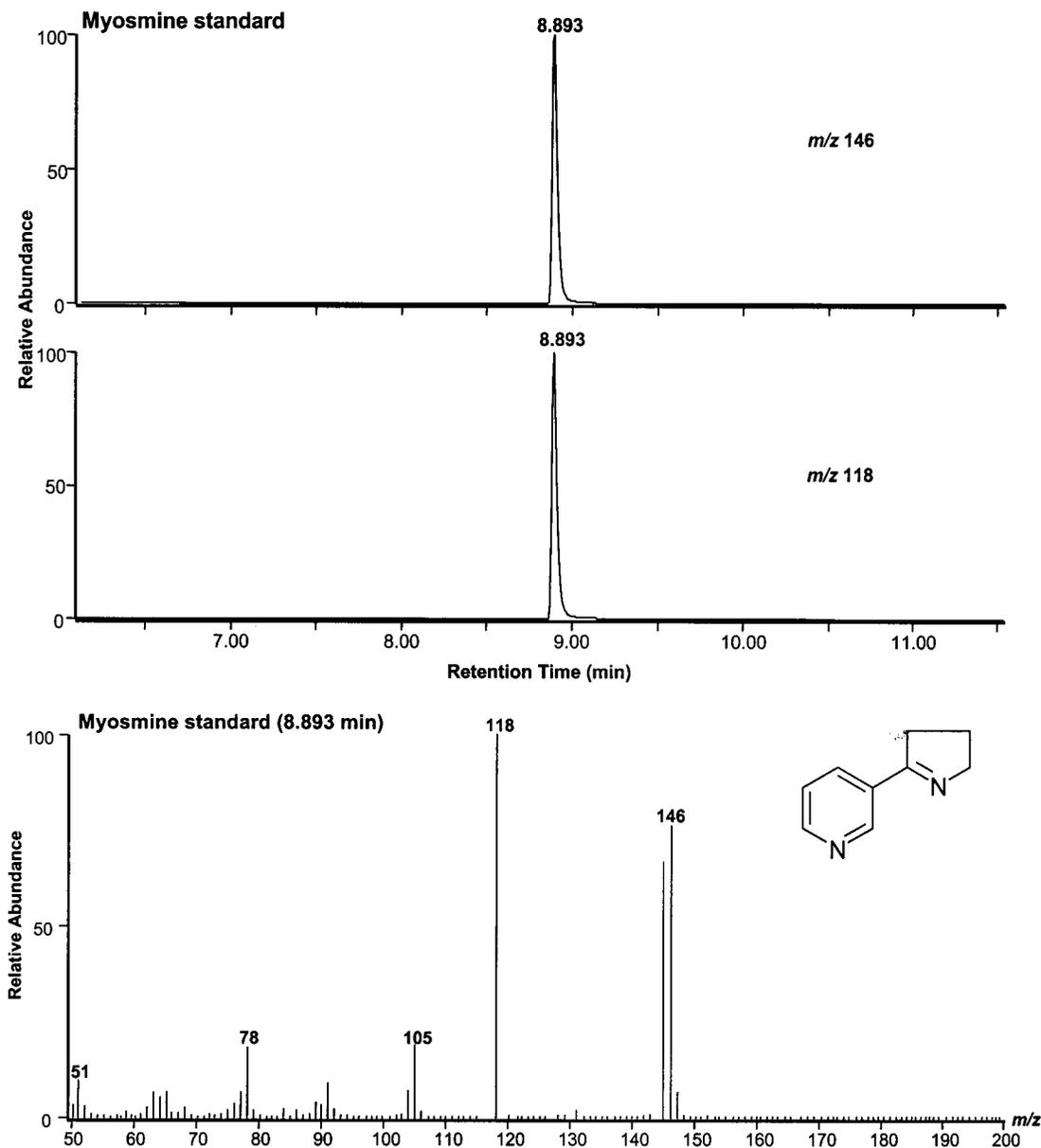
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Figure 1. GC-MS chromatogram and mass spectrum of myosmine standard. The relevant ions *m/z* 118 and 146 are shown in the scan mode at a retention time of 8.893 min.

of their relevance as representatives of important common dietary products. These products are in most cases ready for meal preparation or instant use, for example, fruits or cocoa powder. Other analyzed foods such as rice, maize, wheat flour, millet, and potatoes are normally cooked prior to consumption. The individual cooking processes vary substantially across consumers, and a standard procedure is neither available nor practicable. Therefore, quantification of myosmine was performed using raw material with the exception of potatoes, which were cooked with tap water. Comparative analyses of raw and cooked rice did not show a relevant variation of myosmine content. However, the analyses of cooked, especially starchy, edibles is more difficult to handle than that of the raw material.

Because myosmine is one of the non-steam-volatile bases besides nornicotine and anabasine (*19*), common analytical procedures for food analyses are not practicable, and therefore another analytical method for extraction had to be developed. On the basis of the conversion of myosmine into its amino ketone form by ring opening under acidic conditions, the proposed procedure is applicable to many different food matrices

(*20*). Unfortunately, there are no consolidated findings about the binding of myosmine in biological matrices. In fact, boiling foods such as rice or potatoes in tap water neither reduced nor increased the recovery of myosmine compared to unboiled edibles. After myosmine is transferred into the acidic aqueous phase, the often lipophilic bulk can be separated by centrifugation and filtration. The analytical procedure for starchy and pectin-containing fruits and vegetables required some modification. Whereas acidic hydrolysis of starch led to a sticky mixture of polysaccharides, pectin became gelatinous in the presence of acids and fructose. Under these conditions filters and SPE columns tended to clog rapidly. This problem could be circumvented by further dilution of the extracts and increased rinsing of the SPE columns. Prior to SPE the extracts were adjusted to pH 7 to convert myosmine into its more lipophilic imino form. This allowed further enrichment on the RP-18 material. The eluate could be analyzed by GC-MS without additional purification. Traces of the relevant ions at *m/z* 118, 122, 146, and 150 did not show any compounds interfering with myosmine at the given retention time. It was important to elute
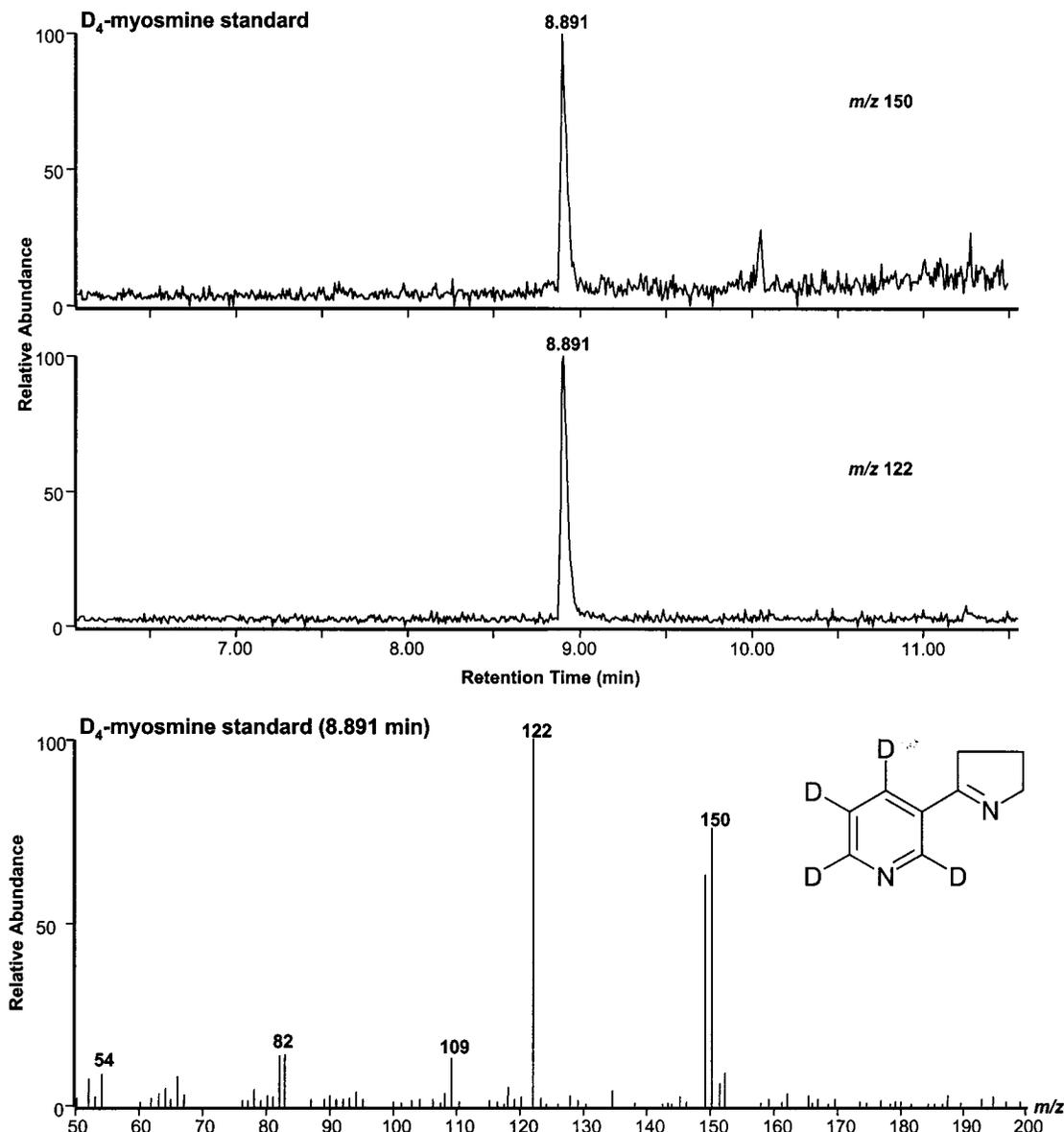
Figure 2. GC-MS chromatogram and mass spectrum of D_4-myosmine standard representing the significant ions *m/z* 122 and 150 in the scan mode at a retention time of 8.891 min.

myosmine from the SPE columns with an apolar solvent such as chloroform. Any addition of more polar solvents such as methanol led to an increase of impurities interfering in the GC-MS analysis. However, for samples such as carrots, containing large amounts of lipophilic pigments coeluting with myosmine from the C_{18} sorbent material, a further cleanup step by TLC may be indicated.

Two aspects of myosmine occurrence were studied in more detail. First, the dependence of myosmine concentrations on the content of milk fat was investigated by analyzing whole milk and cream with 3.5 and 30% fat, respectively. Myosmine contamination clearly increased with higher fat levels. Feeding dairy cows with maize silage, particularly in the winter season, could be one possible source of myosmine in milk. The fate of myosmine in processing of milk products should be investigated in more detail. Second, the concentrations of myosmine in peels not usually eaten were shown to be of the same order of magnitude as compared to the myosmine content in the edible part of fruits and vegetables (**Table 1**). Therefore, the use of peels in human or animal diets may further contribute to the myosmine burden.

The origin of myosmine in the positively tested plants remains speculative. Possible precursors, nicotine and nornicotine (*21*), were not detected with this analytical method. The occurrence of nicotine is not limited to *Nicotiana* spp. Other species such as *Duboisia hopwoodi* and *Equisetum*, *Lycopersicum*, *Lycopodium*, *Sedum*, and *Solanum* species also contain nicotine (*22–25*). The occurrence and amounts of nicotine in edible nightshades (e.g., potato and tomato) has been studied recently in more detail by Siegmund et al. (*26*). In view of the widespread occurrence of myosmine, another unknown biosynthetic pathway for myosmine in plants independent from those of the other tobacco alkaloids should be taken into consideration.

An interesting question is the contribution of dietary uptake of tobacco alkaloids to the total exposure. In the case of nicotine, both Benowitz and Domino concluded, after controversial discussion, that food represents an insignificant source of nicotine exposure in nonsmokers compared to the uptake of this tobacco alkaloid from environmental tobacco smoke (ETS) (*27–29*). This may not hold true for myosmine. Estimations of ETS or dietary exposure through urinary cotinine levels do not register myosmine at all. Another common method to separate
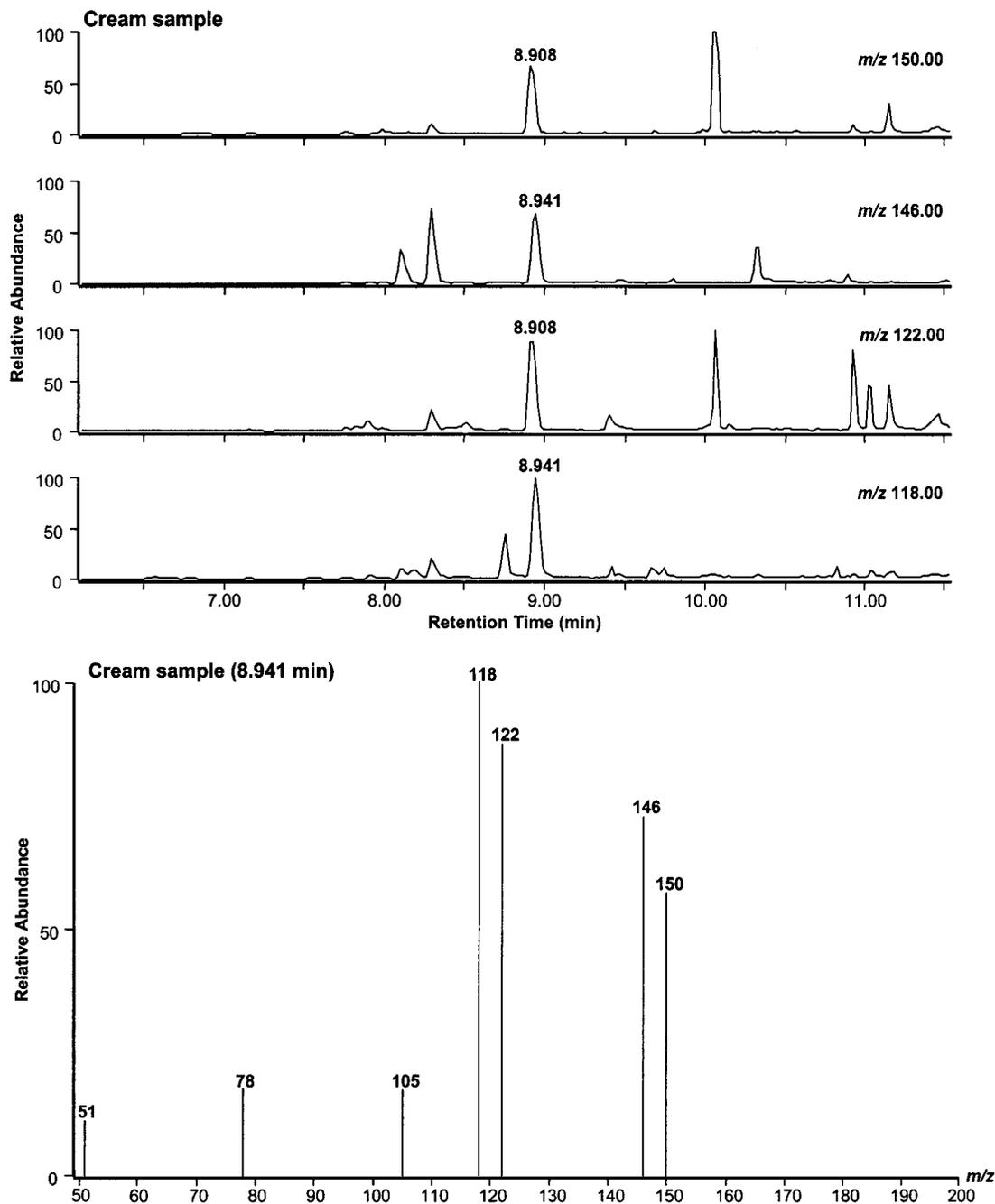
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Figure 3. GC-MS chromatogram and mass spectrum of cream sample extract (SIM mode with relevant ions *m/z* 51, 78, 105, 118, and 146 for myosmine and *m/z* 122 and 150 for D_4-myosmine). The time delay of the retention times is caused by the biological matrix interfering with the analyte.

smokers from nonsmokers or passive smokers via detection of 4-hydroxy-1-(3-pyridyl)-1-butanone-releasing hemoglobin adducts resulting from metabolic activation of tobacco-specific nitrosamines (TSNA) poses other problems (*30*). Studies of pregnant women showed no differences between the adduct levels according to passive smoke exposure (*31*). The 2−3-fold higher levels in smokers compared to nonsmokers are much smaller than expected (*31, 32*). Uptake of dietary myosmine may be a possible reason for this remarkably high background in adduct levels. No direct measurements of myosmine uptake by humans have previously been available. Estimations can be made about myosmine exposure through ETS: The 24 h time-weighted average airborne concentrations range between 0.004 and 0.185 μg of myosmine/m³ (*33*). Therefore, myosmine uptake of 3.65−803.00 μg/year can be estimated for nonsmokers with

low or high ETS exposure (*27*). On the basis of the consumption per head of the tested foods and dietary components in Germany, an average dietary myosmine uptake of 475 μg/year could be calculated (**Table 3**), not including so far unidentified sources as well as individual differences in diet composition. However, taking into account regional varieties in the distribution of consumed edibles, this range would not change dramatically, because a large number of staple foods, which cover eating habits almost worldwide, have been positively tested.

Epidemiologic studies show a correlation between body mass index, eating habits, and the risk of esophageal adenocarcinoma. The genotoxic potential of myosmine has been demonstrated in a bacterial mutation assay (*34*) and by detection of the covalent binding of myosmine to DNA under nitrosation conditions at pH 3−5 (*4*) as well as its DNA-damaging potential

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Table 2. Overview of All Examined Foods and Dietary Components Including Their Membership in the Corresponding Plant Families

myosmine	representative	plant family
detected	hazelnut (*Corylus avellana*)	Betulaceae
	peanut (*Arachis hypogaea*)	Leguminosae
	wheat (*Triticum aestivum*)	Gramineae
	rice (*Oryza sativa*)	Gramineae
	maize (*Zea mays*)	Gramineae
	millet (*Carex panicea* L.)	Gramineae
	potato (*Solanum tuberosum*)	Solanaceae
	tomato (*Solanum lycopersicum*)	Solanaceae
	carrot (*Daucus carota*)	Umbelliferae
	cocoa (*Theobroma cacao*)	Sterculiaceae
	apple (*Malus domestica*)	Rosaceae
	pineapple (*Ananas sativus*)	Bromeliaceae
	kiwi (*Actinidia deliciosa*)	Actinidiaceae
not detected	lettuce (*Lactuca sativa* var. *crispa*)	Compositae
	spinach (*Spinacia oleracea*)	Chenopodiaceae
	cucumber (*Cucumis sativus*)	Cucurbitaceae
	onion (*Allium cepa*)	Liliaceae
	grapes (*Vitis vinifera*)	Vitaceae
	banana (*Musa paradisica*)	Musaceae
	tangerine (*Citrus reticulata*)	Rutaceae
	lemon (*Citrus limon*)	Rutaceae

Table 3. Estimation of Dietary Myosmine Exposure Based on the Tested Foods and Dietary Components and on per Capita Consumption Data of Germany

food	per capita consumption (kg/year)	av myosmine exposure (μg/year)
rice	3.5	2.10
maize	4.5	5.85
wheat flour	58.9	23.56
millet	0.4	0.16
potato	70	238.00
tomato	16.6	9.96
cocoa[a]	1.2	0.96
milk	91.2	109.44
cream	7.8	47.58
carrot	5.9	0.12
apple	31.5	37.80
total		475.53

[a] Based on consumption of cocoa-containing foods such as chocolate assuming an average cocoa content of 20%.

in human lymphocytes and nasal mucosa cells as assessed by the Comet assay (*35*). The occurrence of myosmine in a large number of foods and dietary components may bring both investigations together, implicating myosmine as a possible risk factor for esophageal adenocarcinoma.

ABBREVIATIONS USED

AC, adenocarcinoma; BE, Barrett esophagus; EI, electron impact; ETS, environmental tobacco smoke; GERD, gastroesophageal reflux disease; SCC, squamous cell carcinoma; SPE, solid-phase extraction; TLC, thin layer chromatography; TSNA, tobacco-specific nitrosamine.

ACKNOWLEDGMENT

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